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Enzymatically active lysosomal proteases are associated with amyloid deposits in Alzheimer brain

(cytochemistry/senile plaques/lipofuscin/proteolysis/cathepsins)

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ABSTRACT **The formation of β-amyloid in the brains of individuals with Alzheimer disease requires the proteolytic cleavage of a membrane-associated precursor protein. The proteases that may be involved in this process have not yet been identified. Cathepsins are normally intracellular proteolytic enzymes associated with lysosomes; however, when sections from Alzheimer brains were stained by antisera to cathepsin D and cathepsin B, high levels of immunoreactivity were also detected in senile plaques. Extracellular sites of cathepsin immunoreactivity were not seen in control brains from age-matched individuals without neurologic disease or from patients with Huntington disease or Parkinson disease. *In situ* enzyme histochemistry of cathepsin D and cathepsin B on sections of neocortex using synthetic peptides and protein substrates showed that senile plaques contained the highest levels of enzymatically active cathepsin. At the ultrastructural level, cathepsin immunoreactivity in senile plaques was localized principally to lysosomal dense bodies and lipofuscin granules, which were extracellular. Similar structures were abundant in degenerating neurons of Alzheimer neocortex, and cathepsin-laden neuronal perikarya in various stages of disintegration could be seen within some senile plaques. The high levels of enzymatically competent lysosomal proteases abnormally localized in senile plaques represent evidence for candidate enzymes that may mediate the proteolytic formation of amyloid. We propose that amyloid precursor protein within senile plaques is processed by lysosomal proteases principally derived from degenerating neurons. Escape of cathepsins from the stringently regulated intracellular milieu provides a basis for an abnormal sequence of proteolytic cleavages of accumulating amyloid precursor protein.**

A prominent neuropathologic feature of Alzheimer disease is the deposition of β-amyloid protein within senile plaques. β-Amyloid is composed of a 4.2-kDa polypeptide, the A4 protein, which is generated by proteolytic cleavage of a 70-kDa glycosylated membrane-spanning protein, the amyloid precursor protein (APP) (1, 2). Neither the proteases responsible for APP processing nor the sites in the brain where these proteolytic events occur is known. It has been suggested, however, that most of the abnormal proteolytic processing of APP may take place locally within the senile plaques, since β-amyloid or the A4 peptide is infrequently detected in cells, while APP, a component of membrane-bound compartments of intact neurons (3, 4), is abundant in degenerating neuronal constituents of senile plaques (2, 5–8).

In preliminary immunocytochemical studies of lysosomal proteases in neurons of Alzheimer brain, we observed that cathepsin antigens not only displayed a normal intracellular localization in lysosomes and lysosome-related structures but also were prominent extracellularly in senile plaques (9, 10). Cathepsin immunoreactivity did not appear to colocalize with nonneuronal cells within the plaque (ref. 10; unpublished data), which raised the possibility that degenerating neurons may be the principal source of the antigen. To investigate the significance of these findings, we performed *in situ* enzyme histochemical analyses of cathepsin B (CB) and cathepsin D (CD) activities and immunoelectron microscope studies on the cellular and subcellular localization of these proteases in control and Alzheimer brains. Our studies demonstrated that high levels of enzymatically competent CB and CD are associated with amyloid deposits and are contained within extracellular lipofuscin granules that resemble those in degenerating neurons of Alzheimer brain. These observations form the basis of a possible mechanism for the abnormal processing of the APP to generate β-amyloid.

MATERIALS AND METHODS

Tissues. Postmortem human brains from 10 individuals with a premortem clinical diagnosis of probable Alzheimer disease, 5 individuals with Parkinson disease and Huntington disease, and 10 age-matched (62–78 years old), neurologically normal individuals were used in this study. Brain tissue was obtained from E. D. Bird (McLean Hospital Brain Tissue Resource Center) and from the neuropathology core facility of the Massachusetts Alzheimer's Research Center. Control brains, obtained from patients with no history of neuropsychiatric disease, displayed whole brain weights of ≈1300 g and exhibited negligible microscopic neuropathology (0–2 senile plaques per low power field). Tissue blocks (3 × 1 × 0.4 cm) were dissected from the frontal pole (area 10, prefrontal cortex) of all brains selected and cut into 30-μm-thick vibratome or 20-μm-thick wedge microtome sections.

Serial, adjacent sections from the prefrontal cortices of all brains were prescreened for histopathology and the detection of senile plaques using Nissl (11), modified Gallyas (12), and Bielschowsky(13) stains. Frozen and fixed tissues were used in all immuno- and enzyme cytochemical studies described. The postmortem interval for tissue immersion fixed in cold, 1% phosphate-buffered (0.15 M) formalin was 30 min to 6 hr with a total fixation time of 1 year or less.

Antibodies. Immunocytochemical analyses were performed with a polyclonal antiserum raised in sheep against human brain CD (14). The immunospecificity of this antibody was previously confirmed by immunoblot and immunoadsorption analyses (ref. 10; unpublished data). An antiserum to human liver CB was obtained from ICN or Serotec.

Cytochemical Methods. The protocol for immunocytochemical studies used the avidin-biotin technique of Hsu *et al.* (15) with Vectastain Kits (Vector Laboratories) and diaminobenzidine tetrahydrochloride (DAB) was used as the

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Abbreviations: CD, cathepsin D; CB, cathepsin B; APP, amyloid precursor protein.

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chromagen (10). Immunocytochemical controls consisted of tissue sections incubated in preimmune antisera or in the absence of primary antisera. Thioflavin S histochemistry was used to identify β-amyloid (16). Sections for ultrastructural inspection of immunoreaction product were processed by a pre-embedding staining technique (17). After immunocytochemical staining, selected tissue was postfixed in 1% osmium tetroxide in water for 1 hr at room temperature, rinsed in 0.1 M cacodylate buffer (pH 7.4), dehydrated in ethanols, and embedded in Spurrs resin for 3 days at 37°C. Ultrathin sections were cut and placed on 300-mesh uncoated copper grids and were not poststained.

Enzyme cytochemistry for localization of CB activity was performed by a modified naphthylaminidase method (18) with leucyl-β-naphthylamide used as substrate and fast blue B as the diazonium salt (19). A fluorescent visualization method (20, 21) was also used with benzyloxycarbonyl-alanylarginylarginyl-4-methoxy-2-naphthylamine as the substrate. This method is similar to the above except that 1 mM 5-nitrosalicylaldehyde was used in place of fast blue B. A colorimetric method was used in addition to the fluorescent technique to verify specific lysosomal staining and distinguish this reaction product from lipofuscin autofluorescence. The final pH of the incubation medium in either technique was adjusted to 5.5. Incubations were carried out in a closed chamber system, which allowed extended incubation periods at 37°C. Fresh incubation medium was supplied every 15 min for a total of 2 hr. Tissue sections were counterstained in 1% thioflavin S in water for 15 min and coverslipped immediately using an aqueous mounting medium. Sections preincubated in 100 μM leupeptin for 30 min at 37°C or in an incubation medium at pH 3.2 served as negative controls. For enzyme histochemical detection of CD activity, we modified the technique of Adams and Tuqan (22) by using glass microscope slides (75 × 25 mm) coated in blackened photographic nuclear track emulsion (NBT2) and dipped in a 1% gelatin/10% hemoglobin substrate. Tissue sections were incubated on these slides for a total of 2 hr at 37°C in a humidity chamber and moistened every 10 min with 0.15 M acetate buffer (pH 3.2). After 2 hr, sections were dried and dehydrated through a series of ethanol to xylene and coverslipped in Permount. Proteinase activity was identified as clear areas where the gelatin/hemoglobin substrate had been digested and silver granules had been etched away and washed out. Control sections consisted of tissue preincubated with pepstatin (100 μM) for 30 min at 37°C or sections treated and rinsed during the incubation procedure with a 0.15 M acetate buffer solution (pH 5.5). Tissue sections from mouse neocortex and diencephalon, similarly incubated, were used to optimize the procedure for human tissue and to serve as positive controls in these studies. Perfused fixed rodent tissue exhibited greater activity than immersed fixed, adjacent serial sections. In additional studies with rodent tissue, increasing length of time between death and fixation in cold formalin (between 0 and 12 hr) considerably influenced the sensitivity limits of all histochemical techniques.

RESULTS

As earlier established for CD localization in rodent central nervous system (23), we observed in control and Alzheimer brains that CB and CD immunoreactivities were localized within the lysosomal compartments of neurons, predominantly in perikarya and proximal dendrites (Fig. 1 *A*, *C*, *D*, and *F*). In all 10 Alzheimer brains, however, antisera to both proteases also intensely stained spherical 7- to 20-μm-diameter extracellular lesions, which were identified by thioflavin S histochemistry, Bielschowsky, and Gallyas silver stains as senile plaques (Fig. 1 *B–G*) (10). Elevated levels of cathepsin immunoreactivity were associated with virtually every thioflavin-positive plaque in neocortical tissue sections (Fig. 1*B*). Cathepsin immunostaining within senile plaques appeared as irregularly shaped particles 0.5 to 5 μm in diameter (Fig. 1 *B–F*). Intensely immunostained neuronal perikarya in various stages of degeneration (Fig. 1 *C–G*), some containing neurofibrillary tangles (Fig. 1*G*), were frequently detected in the senile plaques. These cells were identified by using a modified Gallyas silver stain, which included carbol fuchsin to reveal lipids. Degenerating neurons contained large amounts of lipid-positive material that corresponded morphologically to lipofuscin granules (Fig. 1*G*). Neurons near the periphery of the plaque appeared normal or frequently displayed one or more abnormal features, including increased cathepsin immunoreactivity, elevated numbers of lysosomes, neurofibrillary tangles, or eccentric nuclei (Fig. 1 *C–G*). Extracellular deposits of cathepsin immunoreactivity were not observed in any of the control brains (Fig. 2) or in the prefrontal cortices of brains from patients with Huntington disease ($n = 5$) or Parkinson disease ($n = 5$) (data not shown).

To investigate whether or not the cathepsin immunoreactivity within senile plaques reflected the presence of enzymatically competent enzyme, we performed enzyme cytochemical assays of CD and CB on tissue sections and used specific substrates for the two proteases. Preliminary histochemical analyses on freshly perfused fixed mouse brain revealed enzyme cytochemical reactivity at the appropriate

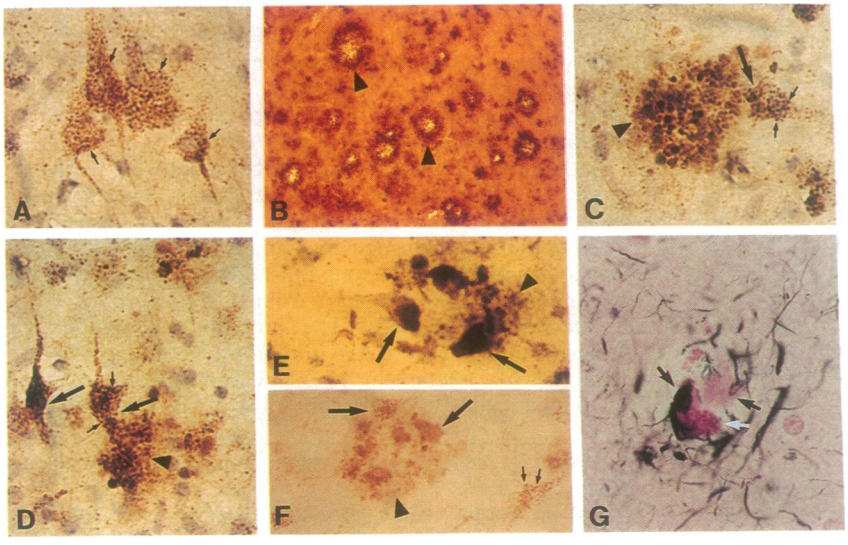

FIG. 1. Staining of neuronal perikarya and senile plaques in the prefrontal cortex of Alzheimer brain. Antiserum directed against CD invariably stained lysosomes within neuronal perikarya (*A*, *C*, *D*, and *F*; small arrows) and extracellular lesions (*B–F*; arrowheads) identified by thioflavin S histofluorescence as senile plaques. Cathepsin-immunoreactive plaques in Alzheimer brains were most numerous within cortical laminae III and V and exhibited thioflavin S-positive cores (*B*). Neuronal perikarya undergoing degeneration (*C–E*; large arrows) were often observed in plaques and, after Gallyas staining (*G*), displayed large amounts of lipid-positive material (white arrow). Note probable degenerated neurons (arrows) within the plaque, one of which contains argyrophilic material (*G*). (*A* and *G*, ×480; *B*, ×180; *C–E*, ×450; *F*, ×270.)

pH optima for CB and CD within neuronal cell bodies of the neocortex and hippocampus (Fig. 3). Although diffusion of the capture agent precluded subcellular localization of cathepsins, the high enzyme activity in certain populations of neuronal perikarya (Fig. 3) accorded with the cellular distribution of these enzymes established by immunocytochemistry (ref. 23; unpublished data). In human brain sections, proteolytic enzyme activities against leucyl-β-naphthylamide, benzyloxycarbonylalanylarginylarginyl-4-methoxy-2-naphthylamine, and gelatin/hemoglobin were barely detectable or absent within neuronal cell bodies. The method and length of fixation appeared to account for the more limited detectability of enzyme activity from human brain sections as compared to optimally prepared mouse brain sections. In Alzheimer brains, however, spherical areas of abundant cytochemical reaction product were observed within the cortical mantle (Fig. 3). Similar areas of enzyme activity were not observed in corresponding sections from any of the normal control, Huntington, or Parkinson brains. Pretreating tissue sections from Alzheimer disease brains with the protease inhibitors leupeptin or pepstatin before incubation at pH 5.5 or 3.2, respectively, eliminated demonstration of proteolytic activity (Fig. 3). Thioflavin S staining of adjacent serial sections in these experiments demonstrated amyloid deposits associated with every area of enzyme cytochemical activity (Fig. 3). CB and CD activities were detected in 25–30% of 50 thioflavin S-positive plaques counted. Studies of a series of formalin-fixed Alzheimer brains varying in postmortem handling conditions confirmed that histochemical detection of cathepsin activity is sensitive to postmortem variables such as postmortem interval and total length of fixation in formalin. Alzheimer brains with the shortest postmortem interval (30 min to 6 hr) and total fixation time in cold 10% phosphate-buffered formalin retained the most enzyme activity (data not shown).

Ultrastructural inspection of sections immunostained with CB or CD antisera established that the protease antigens in senile plaques were not present in intact cells but instead were contained within extracellular membrane-delimited particles (Figs. 4 and 5). Immunoreactivity was distributed relatively evenly or in focal concentrations within these particles. Some of these structures corresponded to dense bodies, spherical or oval organelles (0.3–0.5 μm) bounded by a single limiting membrane and filled with a fine granular material (24). Others correspond to lipofuscin granules, 0.7- to 2.5-μm irregularly shaped complexes bound by a single membrane and containing variable amounts of lipid and pigment material that appeared as loosely arranged, long, single or double, linear elements with ≈8-nm spacing (25). These structures appeared to be the

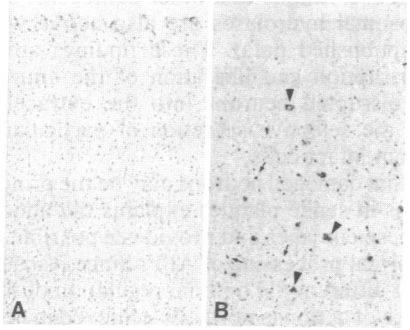

FIG. 2. Prefrontal cortices from a neurologically normal control brain (*A*) and an Alzheimer brain (*B*) immunostained with anti-CD antiserum. In both control and Alzheimer brains, cathepsin immunoreactivity was prominent in lysosomes of neuronal perikaya and was particularly intense within the large pyramidal neurons of "at risk" Alzheimer cell populations (*B*; arrows). In cortex of only Alzheimer brains, cathepsin-positive senile plaques (*B*; arrowheads) were numerous, particularly in laminae III and V. (×70.)

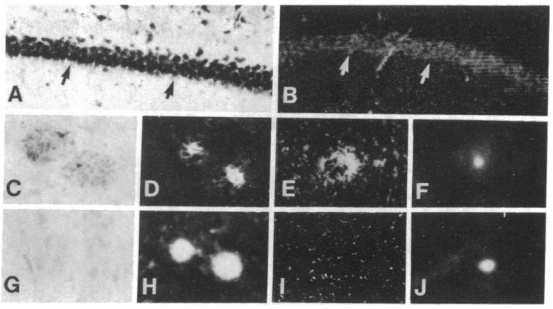

FIG. 3. Histochemical localization of CB and CD activities. Pyramidal cells in hippocampal sections from mouse brain displayed high levels of CB (*A*; arrows) and CD (*B*; arrows) activities. In the prefrontal cortex of Alzheimer brains, senile plaques exhibited abundant CB (*C*) and CD (*E*) activities. The identity of these lesions as senile plaques was confirmed in the same sections by thioflavin S-positive histofluorescence (*D* and *F*). Histochemical activities were eliminated after preincubation with leupeptin (*G*) and pepstatin (*I*). Protease-containing plaques demonstrating inhibitor sensitivity were identified by counterstaining with thioflavin S (*H* and *J*). (*A* and B, ×140; *C–J*, ×300.)

counterpart of the lipophilic profiles detected at the light microscope level with the Gallyas stain. Bundles of amyloid fibrils (diameter, 4–9 μm) were dispersed throughout the areas containing these cathepsin-positive structures. The fibrils were never immunostained.

Lipofuscin granules and dense bodies are usually present in neuronal cell bodies as a consequence of normal senescence; however, they were particularly abundant within degenerating neuronal perikarya in Alzheimer brains (Fig. 5) (unpublished data). These ranged in size from 0.9 to 7.5 μm and exhibited various degrees of cathepsin immunoreactivity. Similar structures were often observed in a confined area adjacent to a nucleus but not surrounded by a plasmalemma (Fig. 5*A*). We identified these regions, which contained abundant aggregates of amyloid fibrils, as the ultrastructural correlates of the senile plaques depicted at the light microscopic level in Fig. 1.

DISCUSSION

These results identify enzymatically competent proteases that exhibit prominent localization extracellularly within senile plaques of Alzheimer brain. The identity of two proteases as CD and CB is based on several observations. Monospecific antisera to each protease, which intensely immunostain senile plaques, selectively stain lysosome-related compartments of cells in control and Alzheimer brain.

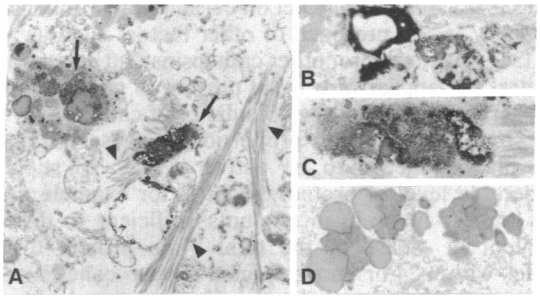

FIG. 4. CD immunoreactivity in senile plaques was localized ultrastructurally within extracellular, membrane-bound organelles that were morphologically typical of lipofuscin granules (arrows in *A*; *B* and *C*). Immunostaining was unevenly distributed throughout the matrix portion of these granules (*A–C*). Aggregates of amyloid fibrils were prominent within plaques (*A*; arrowheads) but were not cathepsin immunoreactive. Lipofuscin granules from unstained tissue sections (*D*) served as negative controls for cytochemical preparations. (*A* and *D*, ×4800; *B*, ×5500; *C*, ×13,000.)

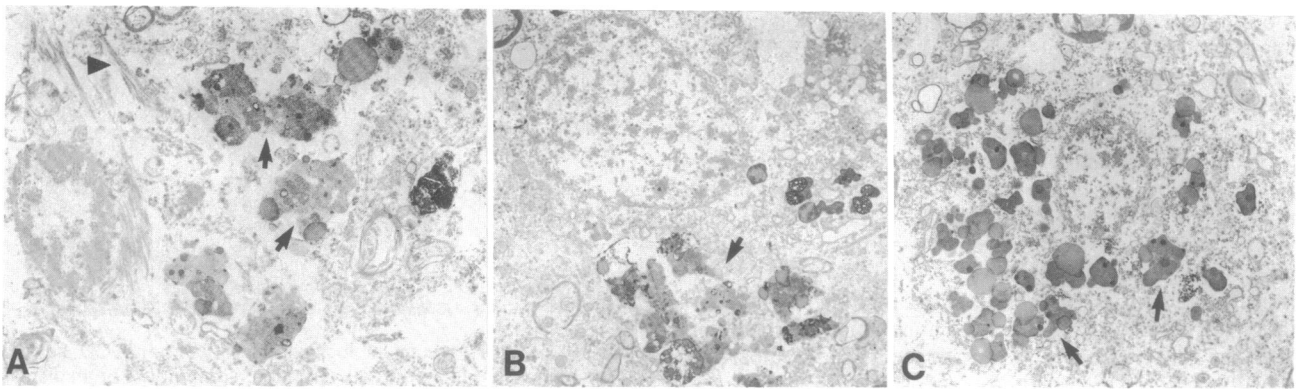

FIG. 5. (*A*) Degenerating neuronal perikarya within senile plaques (e.g., Fig. 1 *C–G*) detected at the ultrastructural level, show degenerative changes, and were associated with abundant, cathepsin-positive lipofuscin granules (arrows) and amyloid fibrils (arrowhead). Lipofuscin is abundant within dying neurons in Alzheimer brain (*B* and *C*) and granules resemble the profiles dispersed within senile plaques (Fig. 1*A*, arrows). (*A*, ×4500; *B*, ×1500; *C*, ×3000.)

CD activity was identified *in situ* by pepstatin-sensitive hydrolysis of a conventional protein substrate at pH 3.2, the pH optimum of CD (1). CB activity was detected by leupeptin-sensitive hydrolysis of specific substrates at pH 5.5 but not pH 3.2 using both colorimetric and fluorescent techniques (26, 27).

Lysosomal proteases are scarce outside cells under normal conditions (27), although cathepsins, particularly CB, may be released in larger amounts from transformed cells when the lysosomal system is stimulated (28, 29). The cathepsin content of cerebrospinal fluid and extracellular fluid from normal mammalian brain is also very low (27). In Alzheimer brain, however, CD and CB were abundant within extracellular components of senile plaques. The activities of these proteases were detectable in 25–30% of the senile plaques even though cathepsin activity in intracellular lysosomes of postmortem human brain was lowered below the limit of sensitivity of the histochemical assays by the fixation and postmortem variables affecting human brain samples. It is reasonable to expect that active cathepsins are present in most senile plaques since cathepsin immunoreactivity associated with thioflavin-positive amyloid deposits exceeded that in intracellular lysosomes, which contain highly active cathepsins when assayed biochemically in fresh brain (27) or histochemically under optimal conditions in rapidly fixed tissues (30–32). Although lysosomal proteases are active *in vivo* in the acidic environment of the lysosome, their activity in senile plaques is more difficult to assess since the pH of compartments containing these enzymes is unknown. Even at physiological pH, however, CB retains considerable activity (33, 34) and CD may act on certain brain proteins (35), particularly in the presence of other factors such as acidic lipids (36). Furthermore, other cathepsins that remain active at neutral pH appear also to be present in plaques (unpublished data). Because of the abundance of cathepsin in brain tissue, only a very small proportion of these enzymes in senile plaques needs to be active *in vivo* to support a considerable level of proteolysis. CD, for example, is normally present in brain in amounts sufficient to digest an equivalent of >95% of the total brain protein to small peptides within 24 hr under optimal *in vitro* conditions (14).

The possibility exists that cathepsins associated with senile plaques are at least partly responsible for the processing of APP to amyloid. Senile plaques contain not only abundant extracellular β-amyloid but also antigenic determinants related to various domains of APP, predominantly within intact and degenerating neurites, (2, 7, 8), which suggests the presence of ample native polypeptide or large fragments capable of being further processed to β-amyloid. Moreover, the size of amyloid deposits correlates with the quantity of associated cathepsin immunoreactivity (37). By contrast, the β-amyloid peptide is rarely observed in neurons (8, 38, 39) and sites of increased APP mRNA transcription and high amyloid deposition are not closely correlated (40, 41). The high activities and range of peptide bond specificities of lysosomal endopeptidases and exopeptidases (27, 42) are well suited for the proteolytic processing of APP. CD and CB, for example, have broad substrate specificities, and the susceptibility of most brain proteins to these proteases (14) contrasts with the relative resistance of β-amyloid to proteolytic enzymes (43–45), including purified human brain CD (R.N., unpublished data).

A likely source of the cathepsin activity within senile plaques is dying neurons and their neurites. Degenerating neurons with disrupted plasma membranes were often identified among the bundles of amyloid fibrils, corroborating the light microscopic identification in senile plaques of neuronal perikarya filled with lipofuscin, cathepsin antigens, and sometimes paired helical filaments. Neuronal perikarya displaying increased cathepsin immunostaining were common within brain regions containing high densities of plaques. The lipofuscin granules and dense bodies in neurons undergoing degeneration had similar morphologies to those in senile plaques. In contrast to neurons, other neural cell types in brain are not heavily immunostained, and the intense cathepsin immunoreactivity within senile plaques does not colocalize with astrocytes and infiltrating inflammatory cells (10). The possibility that cathepsins within senile plaques are derived from intracellular lysosomes is further supported by preliminary observations that other cathepsins and nonproteinase lysosomal hydrolases are also active within senile plaques (unpublished data). These findings are consistent with cell disruption and liberation of the entire lysosome content of disrupted neurons into the extracellular space rather than the selective secretion of particular lysosomal enzymes from intact cells.

The idea that damaged neurons may be the principal source of cathepsins in senile plaques explains the abnormal localization of these enzymes and provides a possible mechanism for the abnormal processing of APP. Since lysosomal hydrolases exhibit different pH optima, regulation of their activity and sequence of action is normally achieved in cells by shifts in intralysosomal pH during autophagy (46, 47). The release of lysosomes, related cathepsin-laden vesicular compartments, and free lysosomal hydrolases from degenerating neurons would disrupt this regulation since the pH of these compartments and of the extracellular space probably differs from that within intracellular lysosomes and is less likely to undergo physiological fluctuations as seen during autophagy. Alterations in the sequence of proteolytic cleavages or in the

nature of peptide bonds cleaved would be expected to favor the generation of abnormal proteolytic fragments of APP, including some that might be amyloidogenic.

One implication of the foregoing hypothesis is that amyloid formation, though not necessarily APP accumulation, largely depends on the degeneration and death of neurons. Some degree of altered APP processing within intact but compromised neurons and their processes, however, cannot be excluded, although β-amyloid fibers or A4 peptide are not commonly detectable within neurons. Normal intracellular processing of APP, possibly within lysosomes (48, 49), is also likely since this polypeptide is expressed in neurons (41, 50, 51). In this regard, we find cathepsin-rich lysosomes to be particularly abundant in affected neurons in Alzheimer brain (ref. 9; Fig. 1). Analogous mechanisms may explain the accumulation of amyloid around the walls of cerebral vessels (52–55), although the common occurrence of β-amyloid at these sites with age and in other neuropathological states could suggest additional etiologic factors. The fact that β-amyloid does not accumulate in the brain parenchyma in other diseases in which neuronal cell death is prominent likely reflects the particular cellular dysfunction that leads to APP accumulation within cells and the pace and specific nature of the cell death process in affected neuronal populations of Alzheimer brain. Finally, our results imply that, if proteolytic fragments of APP have trophic (56, 57) or toxic (58) effects on neurons, then modulating the activity of lysosomal proteases may have therapeutic value in Alzheimer disease.

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